

**The effects of mercuric chloride on
survival, growth, reproduction,
burrowing speed and glutathione
concentrations in the earthworm
species *Eisenia fetida* Savigny**

Master's thesis

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Preface

The work on this thesis was essentially carried out at the Department of Biology, University of Oslo, Norway. Mercury analyses were performed with the help of and on the premises of Holger Technologies, Holmlia, Norway.

With exception of the mercury assays, fairly basic methods were used in this thesis. This made it possible to pursue a more exploratory course, as very little method optimization was needed. On the other hand, some questions surrounding molecular parameters remain unanswered.

Some quick facts from the work on this thesis:

- 1325 analysis of GSH conducted
- 1388 earthworms were killed
- 70 kg soil was contaminated
- 2437 cocoons were counted
- 5322 juveniles were counted
- 10 kg student body mass was lost during the last 6 months of the thesis

Acknowledgements

It takes a village to raise a child, and it seems this also applies to my thesis.

I would like to thank:

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Abstract

The common compost earthworm *Eisenia fetida* was used for toxicity testing of mercury, in a well-characterized agricultural soil obtained from a local field (Ås, Norway). Groups of five or ten clitellate earthworms were exposed to various concentrations of mercury in soil, added in the form of mercuric chloride (HgCl_2) in distilled water. The effects on survival, burrowing behaviour, reproduction, growth, and glutathione levels were recorded. Very low mercury concentration (0.22 mg/kg soil) significantly stimulated cocoon production after two weeks, but higher concentrations reduced it after four weeks (EC_{50} for reproduction was approximately 7.4 mg Hg/kg soil). Protein and glutathione (non-protein thiols) were analyzed by standard spectrophotometric methods. Earthworms exposed to mercury (22 to 740 mg Hg/kg soil for four weeks) experienced a threefold increase in glutathione levels from background levels of approximately 0.62 $\mu\text{mol/g}$ earthworm wet weight. Pre-exposure to mercury (22 mg/kg soil for one week) strongly increased survival in a subsequent standard test carried out one week after the pre-treatment. Differences in cocoon production and growth between pre-exposed and non pre-exposed worms were smaller, though statistically significant. Glutathione levels were similar in pre-exposed and non pre-exposed earthworms, suggesting that increased glutathione levels did not cause the increased survival in pre-exposed animals. Internal concentrations of mercury were determined in both pre-exposed and non pre-exposed earthworms, and followed a one site binding hyperbola. This reached a maximum around 740 mg Hg/kg soil, corresponding to the internal concentrations 139 ± 9 and $165 \pm 16 \mu\text{g Hg/g}$ earthworm wet weight in two independent experiments. The shape of the accumulation curves resembled the shape of the curves for glutathione concentrations plotted against mercury concentrations in soil, suggesting that glutathione levels in earthworms are closely related to internal concentrations of mercury. Therefore, glutathione levels may be a useful biomarker for mercury exposure.

Abbreviations

ANOVA	- Analysis of variance
DDT	- dichloro-diphenyl-trichloroethane
DMA-80	- Direct Mercury Analyzer 80
DTNB	- 5, 5'-Dithiobis(2-nitrobenzoic acid)
GSH	- Reduced glutathione
GSSG	- Oxidized glutathione
HSP	- Heat Shock Protein
mRNA	- messenger Ribonucleic Acid
MT2	- metallothionein 2
NADPH	- Nicotinamide adenine dinucleotide (reduced form)
NO	- Nitric oxide
OECD	- Organization for economic cooperation and development
OH [·]	- Hydroxyl radical
ROS	- Reactive Oxygen Species
SNP	- Sodium Nitroprusside
SOD	- Superoxide dismutase
STD	- Standard deviation
SEM	- Standard error of mean
TCA	- Trichloric acid
TNB	- 5-thio-2nitrobenzoic acid

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1 Introduction

For the last decades mercury has caused environmental concerns, and as a result of recent anthropogenic emissions, these are not likely to diminish in the near future (Hylander and Meili 2003). Mercury is a metal, which exists in three main forms; elemental, inorganic and organic. The elemental form has a relatively high vapour pressure, allowing deposition far from its emission sources. The organometal compound methyl-mercury, produced by sulphur reducing bacteria in sediments and anoxic layers, is biomagnified while the inorganic forms are not (Meili et al. 2003). Not much is known about how mercury affects terrestrial invertebrates (Lock and Janssen 2001). This thesis aims to broaden the understanding of how mercury influences earthworm survival, reproduction, growth, burrowing speed and glutathione levels.

1.1 Earthworms in terrestrial ecotoxicological testing

Earthworms constitute a major part of the animal biomass in terrestrial ecosystems. They have important functions for soil fertility and conservation, and are also a source of food for other organisms. The worms eat and crush an average of 300 tonnes soil/ha each year, thereby mixing mineral layers with organic compounds to produce soil crumbs (Bouché 1992). They produce channels which promote respiration of soils and infiltration of water. Also, they are detritivores, and contribute to the breakdown of organic matter such as leaves, plants and decaying animal remnants (Roberts and Dorough 1985). A healthy population of earthworms is of obvious importance given their key role in soil maintenance (Reinecke 1992). Thus, any effects on survival, reproduction, growth or behaviour (e.g. burial time) are of interest when assessing the effects of a chemical on terrestrial ecosystems.

The effects of mercury on earthworms ultimately depend on the tissue concentration. Soil contaminants are taken up by worms either by diffusion from pore water across the surface, or by absorption from decomposed food in their intestines (Sims and Gerard 1985; Stenersen 1992). The concentration of mercury in soil pore water is a strong indication of the bioavailability, as uptake in earthworms mainly comes from this compartment (Janssen et al. 1997). The amount of contaminants in soil pore water

is governed by abiotic factors such as soil organic content, cation exchange capacity, pore water pH and ionic strength, with pH and cation exchange capacity being the most important for heavy metals (Van Gestel 1992).

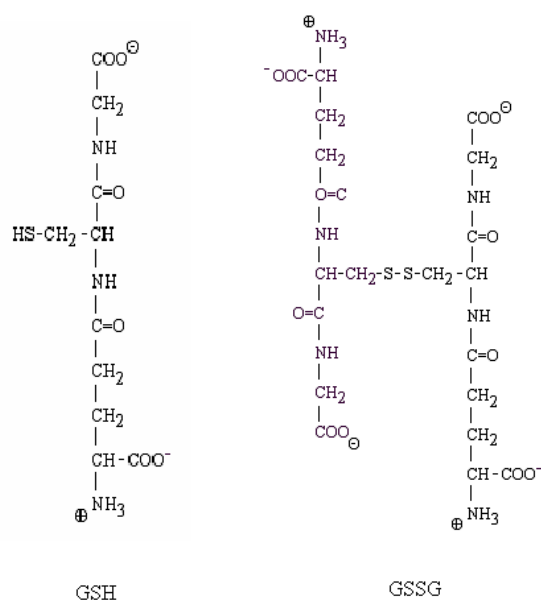
Some pesticides and metals accumulate in earthworm tissues without harming the worms themselves, but may affect predators (Fischer and Koszorus 1992; Reinecke 1992). The best known example of this provided the title for Rachel Carson's famous book "Silent Spring" from 1962, where robins were poisoned after eating earthworms that had accumulated DDT.

Earthworms are segmented organisms within the class Oligochaeta. The family Lumbricidae contains many of the most common earthworm species, including the nightcrawler *Lumbricus terrestris* and the manure worm *E. fetida* (Roberts and Dorough 1985). Earthworms possess several qualities that make them suitable indicators of terrestrial ecotoxicity; they are numerous, relatively large and easy to collect and identify. Also, they are well dispersed, not very mobile, and are in full contact with and consume a large part of the soil. *E. fetida* can easily be cultivated under laboratory conditions, but this hardness makes extrapolation to other species difficult.

1.2 Glutathione

Glutathione (GSH) is a tripeptide comprised of glycine, cysteine and glutamic acid, the latter being linked to cysteine via the γ -carboxyl group (Klaasen 2001). In the cell, reduced glutathione and oxidized glutathione (GSSG) constitute the free glutathione, of which GSH makes up 99.5%

(Anderson 1985). GSH is an antioxidant and reduces reactive oxygen species (ROS) formed during cellular metabolism and respiratory bursts. It is also important in the maintenance of sulfhydryl groups of various enzymes and proteins, keeping them in the reduced form, a requirement for their normal function (Pastore et al. 2003).



GSH is the major intracellular non-protein free thiol in most species, including animals, plants and microorganisms (Kosower and Kosower 1989). When donating an electron, the tripeptide is oxidized to GSSG, which is again recycled to GSH via the NADPH dependent enzyme glutathione reductase (Pastore et al. 2003). Irreversible cell damage such as membrane lipid oxidation supervenes when GSH is depleted (Pastore et al. 2003; Pompella et al. 2003). Glutathione also plays a role in the trafficking of metal ions such as divalent mercury (Pompella et al. 2003).

Kurozumi et al.'s (2002 and 2003) work on mouse macrophage like cell cultures showed that oxidative stress, initiated by sodium nitroprusside (SNP), increases the expression of genes involved in glutathione production. SNP contributes to oxidative stress through its ability to act as a nitric oxide (NO) donor and its iron moiety, which produces hydroxyl radicals (OH^\cdot) via the Fenton reaction. OH^\cdot and NO are reactive oxygen species and lead to an increased consumption of GSH. This may happen directly, through interaction with GSH, or indirectly, through oxidation of other antioxidants such as ascorbic acid, which is reconstituted by a GSH-dependent enzyme (Pastore et al. 2003). Kurozumi and Kojima (Kurozumi and Kojima 2002) found a significant increase of GSH production after exposure to SNP, and also after γ -ray irradiation. The increase was accompanied by an increase in expression of γ -glutamylcystein synthetase mRNA, which is a rate limiting enzyme in GSH production (Kurozumi et al. 2003). This is considered an adaptive response of living cells to ROS (Kurozumi and Kojima 2002; Kurozumi et al. 2003).

The onset of oxidative stress in earthworms from exposure to fluoride, which inhibits superoxide dismutase (SOD), has been shown to increase the levels of glutathione in earthworms (Lawson 2003). However, Saint Denis et al. (1999 and 2001) and Ribera et al. (2001) report that total glutathione levels are unchanged in earthworms after exposure to benzo(a)pyrene, lead acetate and carbaryl.

Glutathione is generally considered a cellular protagonist, but may also contribute to metal induced oxidative stress by donating electrons to metals such as iron and copper. Electrons can in turn be transferred to molecular oxygen, generating

superoxide anion, which easily dismutates into the pro-oxidant H_2O_2 (Pompella et al. 2003).

1.3 Biological effects of mercury

Ionic mercury, Hg^{2+} , has a high affinity for sulfhydryl groups (-SH) and will form nearly insoluble salts, which is the reason for the high toxicity. It can bind to cellular components such as enzymes in the cytosol and various organelles. Proteins with sulfhydryl groups, such as membrane ATPases, are the most susceptible (Stenersen 2004). In addition, ionic mercury can also react with amino-, phosphoryl- and carboxyl-groups (Klaasen 2001).

Some -SH containing proteins and peptides, like glutathione and metallothioneins, protect cells from electrophiles such as mercury. Mercury forms conjugates with glutathione and is probably excreted in the form of $\text{Hg}(\text{GS})_2$, which is the most stable (Foulkes 1993; Lash 1998; Stricks and Kolthoff 1953; Taniguchi et al.; 1989; Zalups 1995). Significantly reduced levels of glutathione in the kidneys of rats exposed to high doses of mercuric chloride have been reported (Nielsen et al. 1991) and decreased levels in mitochondria have also been found (Lund et al. 1993).

Many metals are sequestered by metallothioneins. Homa et al. (2005) showed that in response to various metals, earthworm immune cells increase their levels of mRNA coding for metallothionein 2 (MT2), HSP 70 and HSP 72 after a 3 day cutaneous exposure.

Glutathione is an important part of the cellular defence against ROS, and it is plausible that its production is coupled to the red-ox state of earthworms. A mercury challenge to cells will consume glutathione, thereby reducing the levels of intracellular antioxidants. Lund et al. (1993) propose that mercury leads to increased H_2O_2 formation in the mitochondrial respiratory chain. H_2O_2 is metabolized by mitochondrial GSH peroxidase, leading to formation of GSSG in rat kidney mitochondria. Furthermore, mercury is known to reduce the activities of SOD, catalase and glutathione peroxidase (Stohs and Bagchi 1995). These effects increase the level of oxidative stress, and may activate defence mechanisms, such as increased

GSH levels via an increase of γ -glutamylcystein synthetase or glutathione reductase. However, to the best of my knowledge, this has not yet been studied in earthworms exposed to mercury.

1.4 Scope of investigation

This investigation focused on selected responses of earthworms (*E. fetida*) to mercury exposure. The high affinity of mercury for sulfhydryl groups suggests that its presence will initially deplete tissues of GSH. It is known that metal exposure leads to an increased production of several proteins important for the protection of vital cell functions. Whether mercury will deplete or increase GSH levels in earthworms is not known, and results from other studies on substances known to cause oxidative stress are contradictory. If GSH levels are closely related to mercury accumulation, it may serve as an unspecific biomarker of oxidative stress derived from mercury.

In response to low levels of contaminant exposure, increased growth, survival and reproduction of test animals has been observed in several studies. This phenomenon is called hormesis (Calabrese 2005). Maintaining GSH levels is essential for cell vitality and organism health. Mercury exposure may increase GSH levels to an extent that is overcompensating for the depletion due to detoxication. This will add to the general population of electrophile scavengers and thereby increase the cellular defence. Thus, it can be speculated that low levels of mercury exposure can increase the vitality of cells and organisms. If low levels of mercury cause glutathione levels to rise without adversely affecting reproduction, survival or growth, it may be an indication of hormesis. Also, an increased resistance to mercury as a result of pre-exposure may indicate the same phenomenon.

The aim of the investigation was therefore threefold:

- 1) To investigate whether glutathione can serve as a biomarker for mercury exposure in earthworms compared to the more established markers survival, reproduction, growth and accumulation of mercury.
- 2) To investigate parameters related to hormesis, by looking at biochemical responses to exposure to a variety of mercury levels and by using several

exposure regimes (e.g. short term vs. long term exposure or pre-treatment), while at the same time measuring vitality of the organisms.

- 3) To determine various sub-lethal doses for further research on parameters such as reproduction, survival, glutathione levels and growth.

2 Materials and methods

2.1 Materials

2.1.1 Chemicals

All chemicals that were used were of p.a. (pro analysis) grade of purity.

Sigma, Oslo, Norway:

Bovine serum albumine	(99% pure)
Bradford reagent	
5, 5'-Dithiobis(2-nitrobenzoic acid)	(99% pure)
Glutathione, reduced form	(99% pure)
Guanidine hydrochloride	(99% pure)
Trichloroacetic acid	(99% pure)

Merck, Darmstad, Germany:

CaCl ₂ * 2 H ₂ O	(99,5% pure)
KHCO ₃	(99,5% pure)
MgSO ₄ * 7 H ₂ O	(99,5% pure)
Na ₂ HPO ₄ * 2 H ₂ O	(99% pure)
NaH ₂ PO ₄ * H ₂ O	(99,5% pure)
NaHCO ₃	(99,5% pure)
NaOH	(99% pure)

Ferak labs, Berlin, Germany:

HgCl ₂	(99.5% pure)
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Angus, New York, USA:

Tris(hydroxymethyl)-aminomethane (Ultra Pure)

2.1.2 Test organisms

Clitellate specimens from the species *Eisenia fetida*, with an average weight of 0.25 g, were used as test organisms in the experiments. The population was collected from a

local field at Ås in June 2001. It was cultured at room temperature in boxes containing commercial plant-soil from OASEN garden centre (Oslo, Norway) and had a pH 5.5 to 6.5 and consisted of 95% peat moss and 5% sand. Low genetic diversity was expected in the inbred population. The earthworms were provided with dry and ground horse manure as food. The manure was dried to prevent formation of mould, which appears when it is stored wet, and to evaporate ammonia absorbed into the manure from urine in the stables.

2.1.3 Test substrates

A sandy loam was collected from a research field in Ås, Norway. The soil was dried at 60°C in a Termaks warming cabinet (Bergen, Norway), and then sieved through a 2 mm mesh to remove stones and plant material. The soil was composed of 76.1% sand (63-2000 µm), 14.6% silt (2-63 µm) and 9.3% clay (< 2 µm), and the total carbon content was 1.6 %. The soil pH and total cationic exchange capacity were 6.2, and 120 mmol (+) kg⁻¹, respectively. This soil was used as substrate in all the experiments, except from the toxicology testing in an artificial substrate, see section 2.3.7, where cross-linked dextran (Sephadex G-100 produced by Pharmacia, Uppsala, Sweden) was used. The gel did not have absorption sites for mercury, thus the bioavailability of mercury was equal to the concentration added.

Mercury solutions were prepared by diluting a stem solution of 5 mg HgCl₂/ml in water to the appropriate concentrations for the experiments. These were thoroughly mixed with soil to ensure an even distribution. For the sub-chronic and chronic low-level exposures, the amount of solution was equivalent to 20% of the dry weight of the soil. It was increased to 25% in later experiments because of concerns that 20% did not provide the worms with enough moisture. In the experiment where Sephadex was used, the mercury solution was mixed with a pore water mimicking saline.

Soil pore water mimicking solution

In 1 Liter distilled water:

20 mg	KHCO ₃
200 mg	CaCl ₂ * 2 H ₂ O
180 mg	MgSO ₄ * 7 H ₂ O
100 mg	NaHCO ₃

2.2 Endpoints

The endpoints described below were used in some, but not all experiments. The combinations of endpoints relevant for the individual experiments are described in sub-chapter 2.3.

2.2.1 Survival

Survival was assayed by counting living worms at the end of each test. No attempt was made to predict their viability, as it was observed that badly injured earthworms could recover. Instead of excluding worms in bad shape, their physical conditions were recorded.

2.2.2 Growth

Growth was measured as weight change in earthworms, given as percent change relative to weight before exposure. Prior to the experiments, the weight of the earthworms in each box was recorded collectively to the nearest 0.01 g, with a Sartorius BP2100 S (Göttingen, Germany) scale. After the experiments this was repeated, and in addition the individual weights of the worms were recorded for use in relevant assays. To avoid bias in the measurements, earthworms were depurated on filter paper for 24 hours. This was not performed for the acute exposure and recovery trial (see section 2.3.4), as the experimental design involved sampling at exposure times less than 24 hours. Artificial soil pore water was used instead of tap water in an attempt to minimize the stress to the earthworms while on filter paper. Tap water was only used in the sub-chronic and chronic low-level exposures.

2.2.3 Reproduction

The reproduction was assayed by counting the number of cocoons after one reproductive cycle (i.e. four weeks). Hatchlings were counted in the low-level experiments to investigate if a normal amount of the cocoons hatched to give living juveniles, but this was not pursued in later experiments due to time constraints.

2.2.4 Burial time in soil

In this test groups of 5 earthworms were spread out on top of the soil, and the time until they were completely immersed was recorded. The test was terminated after a maximum of 8 minutes. The burial times of the exposed earthworms was compared to controls to evaluate the effect of mercury.

It was observed that the earthworms that did not disappear into the soil within 8 minutes were not capable of moving into the soil at all. Burial times for these could not be obtained, and to avoid losing data, the inverse of time was used. Earthworms that were unable to burrow were set at 0, while the speed of the ones that were able was given as $(\text{burial time})^{-1}$.

Water corresponding to 20% of the dry weight of the soil was added. Experience showed a tendency of the soil to aggregate into a single large lump when more water was added, which the earthworms did not burrow into.

2.2.5 Tissue mercury concentrations

To measure the mercury concentration in the tissue, 100 μl of the homogenate for the protein and glutathione assays was set aside and stored at -80°C until analysis. See section 2.2.7 for description of the homogenization process.

Mercury content in earthworms and soil pore water was analyzed with the Direct Mercury Analyzer, DMA 80 (Milestone, Connecticut, USA) with the help of Holger Technologies in Norway. This is an automated atomic absorbance spectrophotometer capable of processing 40 samples within 5 hours as shown schematically in Fig 2.1. The DMA-80 prepared the sample for analysis by drying it at 300°C for a few minutes and then heating it to 850°C , at which the mercury leaves the sample as a gas. A catalyst then oxidizes it to prevent interferences. The gas is led into a gold filter, which amalgamates the mercury and retains it until the other substances have passed out of the system. The gold filter is heated and the mercury is released into an atomic absorbance spectrophotometer that measures the mercury at 254 nm. This operates in two ranges; a low range spanning from 0 to 35 ng Hg and a high range that spans 35 to 600 ng Hg. The switch between these ranges is automated, so samples in both the

high and low end of the scale are accurately measured and equally reliable. The calibration was done using $HgNO_3$ standard-solution diluted to 20 ng Hg/ml (20 μ L of a 1mg/ml solution in 10 ml distilled water).

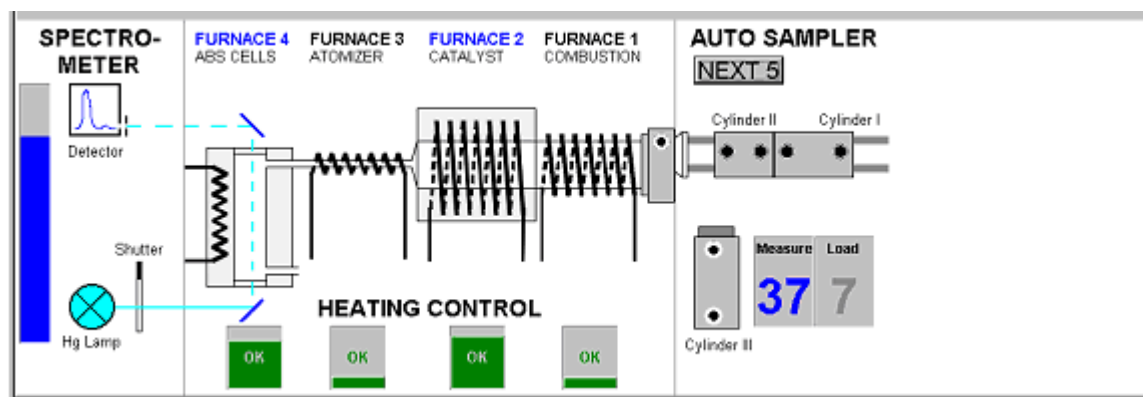


Fig 2.1 – The system software interface, showing the sequence of events during analysis of mercury with DMA-80.

2.2.6 Mercury concentration in soil pore water.

The pore water was extracted by centrifugation, using a 5 ml surety column from Evergreen scientific (number 208-3193-020). The column was packed with 4.0 g soil, inserted into a centrifuge tube and spun at 16.000 g for 5 minutes. The column was removed and the tube was centrifuged once more under the same conditions, to counteract any stirring of fine particles during the removal of the filter. The filtrates were then extracted and transferred to 1.5 ml cryo-tubes and stored at -80°C until they could be analyzed using the DMA-80 as described in section 2.2.5.

2.2.7 Tissue glutathione and total protein levels

The earthworms were homogenized individually with an ultra turrax in the ratio 1:4 with 5% TCA. The samples were centrifuged at 16.000 g in a cooled Heraeus 3 S-R multifuge (Kendro Laboratory Products, Hanau, Germany) for 30 minutes, after which 0.5 ml of the now yellow and transparent fluid was used for analysis of non-protein thiols (i.e. glutathione).

The pellet acquired during centrifugation was used in the protein-assays. It was washed with 1 ml 25% TCA and centrifuged. The new pellet was dissolved in 1 ml

6M guanidinium hydrochloride (GH). The pellet did not completely dissolve, as it contained some body parts that were not broken down by acid or mechanical treatment. The solution was centrifuged for 30 minutes. The protein-assays represent the GH-soluble portion of acid-precipitated proteins in the earthworms.

2.2.7.1 Glutathione assay

The glutathione assays were based on the method measurement of tissue sulfhydryl groups described by Ellman (1959). 5, 5'-Dithiobis(2-nitrobenzoic acid) (DTNB) was used to measure the level of glutathione in the earthworms. It is a sensitive reagent for measuring the free sulfhydryl content in proteins, peptides, and tissues. DTNB reacts with two GSH molecules to yield GSSG and a yellow reaction product 5-thio-2-nitrobenzoic acid (TNB), see figure 2.2. TNB has an absorbance maximum at 412 nm, and the absorption reflects the concentration of glutathione.

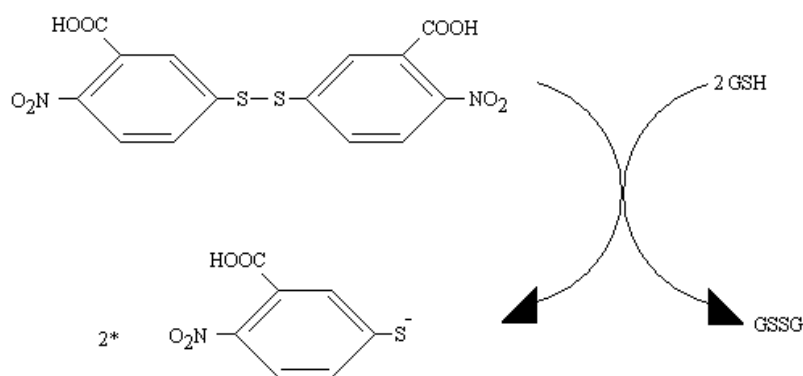


Fig 2.2 – The reaction between DTNB and GSH, producing 2 TNB molecules and oxidizing two GSH molecules.

A glutathione reducing enzyme, glutathione reductase, which recycles GSSG to GSH after reaction with DTNB is often used for glutathione analysis (Pastore et al. 2003). This would have allowed measurements of smaller quantities of GSH. However, this was recognized to be time consuming and superfluous, based on the large number of samples to be assayed and the relatively large sample volumes.

A non-enzymatic measurement of glutathione is more vulnerable to interference from non-protein thiols that were not precipitated by TCA, such as cysteine and coenzyme A. But, as GSH is the major intracellular non-protein thiol in most species, this contribution can be neglected (Kosower and Kosower 1989). In addition, non-protein

thiols play a potential part in the cellular defence against electrophiles. Any impact they may have had on the assay would therefore not have given an erroneous picture of the sulfhydryl based cellular defences.

The original setup for the glutathione assay was only used in the sub-chronic intermediate-level test. The need for adjustment was recognized with the observation of high levels of variance in the readouts, and that the controls often had higher readouts than the samples. The time from neutralizing the sample until DTNB was added was reduced to 10 seconds, to avoid the time dependent oxidation of glutathione. The earthworm homogenates were diluted in acid in the ratio 1:4 instead of 1:9, since a lower dilution was expected to increase the readouts. In addition the concentration of DTNB was reduced from 2 mg DTNB/ml Tris to 0.2 mg DTNB/ml Tris, to avoid the impact from fluctuations in absorbance of the DTNB control.

Concerns were raised that the low amounts of DTNB would not be sufficient to measure the total glutathione content in the samples, and thereby affect the net readout of glutathione. 5 earthworms were homogenized in 5% TCA in the ratio 1:4, and assayed for glutathione with increasing levels of DTNB. It was concluded that the concentration of DTNB did not affect the net readout of glutathione (Fig 2.3).

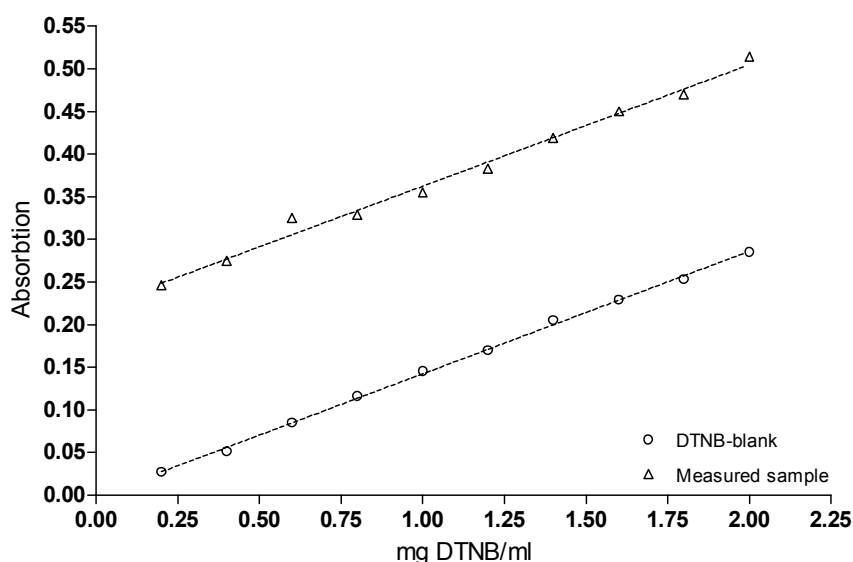


Fig 2.3 – Influence of DTNB on glutathione absorbance. Net glutathione absorbance equals the distance between the two sloping lines. This is not affected by the amount of DTNB, suggesting accurate measurements also at low glutathione concentrations.

Samples were prepared for analysis by adding 0.17 ml 1M Tris solution per 0.5 ml of the samples in order to neutralize them. Two blanks were used, one for measuring the absorbance contributed by the sample and another for the absorbance from the DTNB solution. The two blanks and the sample assays were mixed as shown in Table 1. The DTNB and sample were incubated for 10 minutes before the absorbance at 405 nm was measured with a spectrophotometer (DU®-62, Beckman, Fullerton, USA). The absorbance of both blanks were added up and subtracted from those measured in the sample assays.

Table 1 – Volumes added to the cuvettes.

	DTNB blank	Sample blank	Assayed sample
Phosphate buffer	1.0 ml	1.0 ml	0.8 ml
DTNB solution	0.2 ml	-	0.2 ml
Sample	-	0.2 ml	0.2 ml
Total volume	1.2 ml	1.2 ml	1.2 ml

The DTNB solution was made by weighing in 2 mg DTNB to the nearest 0.0001 g on a Mettler Toledo AG204 (Greifensee, Switzerland) and mixing with 10 ml of 0.05M, pH 7.5, Tris buffer. The phosphate buffer was prepared by mixing $\text{Na}_2\text{HPO}_4 \cdot 2 \text{H}_2\text{O}$ and $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, each at 0.2M, until pH 7.5 was obtained, measured with an Orion 720 pH-meter (Boston, USA). Standard curves for GSH were made prior to analysis in each experiment.

The maximal absorbance wavelength for DTNB is 412 nm, but 405 nm was used as a compromise to be able to compare the results with a second spectrophotometer, an Emax™ plate-reader from Molecular Devices, California, USA. This did not appreciably affect the results.

2.2.7.2 Protein assay

The effect of mercury may have caused some individuals to lose or retain water during the exposure. In the glutathione assays, the earthworms were diluted in proportion to weight, and this could have affected the levels measured. The measurement of total protein content will not be affected by dilution during

homogenization and the protein assay was performed to validate the results from the glutathione assay.

The Bradford reagent (Bradford 1976) was used to measure total acid-precipitated protein content in the earthworm sample, by using the procedure proposed by Sigma-Aldrich in the technical bulletin for their Bradford-reagent (Sigma-Aldrich, Saint-Louis, USA). A 50 µl sample, prepared as described in section 2.2.7, was mixed with 1.5 ml Bradford reagent in 1.5 ml cuvettes. The control was made by substituting the sample with deionised water. This method was also subject to a small revision as readouts were very high and bordered on the edge of what the instrument was capable of detecting. For the high-level exposures, the amount of sample was reduced to 7.5 µl and the remaining volume was replaced with distilled water.

2.3 Exposure strategies

A number of experiments were performed to evaluate how earthworms responded to different exposure concentrations of mercury, duration of exposure and pre-treatment with the substance. Most of the experiments conducted were based on the OECD guideline for earthworm reproduction (OECD 2000). Exceptions were the experiments 2.3.2, 2.3.4 and 2.3.7. Also, a few adjustments were made to the OECD test. The artificial soil recommended was substituted with a readily available and well characterized agricultural soil, see section 2.1.3 and the exposure period lasted only two weeks in 2.3.1, instead of the four weeks recommended.

2.3.1 Sub-chronic low-level exposure

The experiment was carried out in order to investigate if low concentrations of mercury would have adverse effects on the earthworms after two weeks exposure. The concentrations used were 0, 0.074, 0.22, 0.74, 2.2 and 7.4 mg Hg/kg. A total number of 240 earthworms were randomly distributed into groups of 10, and exposed in 0.8 l containers with four replicates. The cocoons were counted at the end of the exposure and reintroduced to the soil. The hatchlings were counted six weeks after removal of the adults. The assayed endpoints were earthworm growth, reproduction and survival.

2.3.2 Chronic low-level exposure

The experiment was performed to find out if the same concentrations of mercury as used in the sub-chronic low-level exposure would have stronger effects after four weeks exposure. The experimental setup and endpoints assayed were the same as used in 2.3.1, but the cocoons were counted after the four weeks exposure, and juveniles were counted eight weeks after removal of the adults.

2.3.3 Sub-chronic intermediate-level exposure

The experiment was carried out to investigate if exposure to intermediate concentrations of mercury for ten days would have stronger adverse effects on earthworms than the sub-chronic and chronic low-level exposures. The exposure concentrations were 0, 7.4, 18.5, 37 and 74 mg Hg/kg soil. The lowest concentration in this experiment was the same as the highest concentration in 2.3.1 and 2.3.2.

A total of 120 earthworms were randomly distributed into groups of 6 and exposed in 0.1 l boxes containing 60 g dry substrate. There were 6 replicates in the control groups, 3 replicates in the two lowest concentrations and 4 replicates in the two highest concentrations. The substrate was a 1:1 volume mixture of soil and horse manure. The new substrate was used to counteract the observed tendency of the worms to crawl into the manure used as food when concentrations of mercury were high. Added water corresponded to 40% of the dry weight, because manure has lower weight per volume than soil.

The boxes were checked daily for dead earthworms. After the exposure period, the earthworms were weighed and stored in eppendorf-tubes at -80°C. The assayed endpoints were tissue mercury level, burial time in soil, growth, survival, tissue glutathione level and total protein content.

2.3.4 Acute exposure and subsequent recovery trial

The experiment was performed to investigate the effects on earthworms caused by acute exposure to a high concentration of mercury, and their ability to rehabilitate. A total of 200 earthworms were used, divided 1:1 in controls and exposed worms, and with 4 replicates. While the controls were kept in clean soil, the exposed earthworms were subjected to 148 mg Hg/kg for 24 hours. This concentration had already been observed not to cause mortality in earthworms exposed for 24 hours. After exposure, the earthworms were transferred to clean plant-soil. Samples were extracted at set intervals; 0, 12, 48 and 96 hours and two weeks after transfer. The endpoints assayed were; tissue mercury level, tissue glutathione level, total protein content, burrowing speed, growth and survival.

2.3.5 Chronic high-level exposure

The experiment was carried out to investigate the effects on earthworms after four weeks exposure to high concentrations of mercury. The concentrations of 0, 7.4, 22, 74, 220 and 740 mg Hg/kg soil were used. A total of 120 earthworms were exposed, divided in groups of 5, and 4 replicates were used per treatment. The boxes were inspected regularly, and dead earthworms were removed. The assayed endpoints were tissue mercury level, burial time in soil, reproduction, growth and survival.

2.3.6 Effect of pre-exposure to mercury

This experiment was performed to determine whether pre-exposure to mercury for one week would have negative or positive effects on earthworms when exposed to high concentrations of mercury for four weeks. The mercury concentration used for pre-exposure had been observed not to cause lethal effects to earthworms, but at the same time arrest cocoon production.

A total of 360 earthworms were used, and half of these were pre-exposed to 22 mg Hg/kg soil. The rest were kept in clean soil. After the one week, the pre-exposed and non pre-exposed worms were transferred to clean plant-soil with ample food for one more week. The earthworms were again transferred, to soil spiked with 0, 7.4, 22, 74,

220 or 740 mg Hg/kg soil. The rest of the procedure was carried out as described in section 2.3.5.

The surviving earthworms and cocoons were counted at test termination, and the worms were weighed and frozen. The assayed endpoints were tissue mercury level, tissue glutathione level, total protein content, growth, reproduction and survival.

2.3.7 Earthworm toxicology testing in an artificial substrate (Sephadex)

The aim of the experiment was to investigate if Sephadex G-100 could be used as an inert substrate for acute toxicity testing, instead of soil. Lethal concentrations of mercury in Sephadex are expected to be lower than in soil, due to the lack of absorption sites for mercury.

A total of 132 earthworms were exposed to 0, 0.74, 1.5, 3.0, 4.4, 5.9, 6.7, 7.0 and 7.4 mg Hg/ml artificial pore water. There were six replicates of the control and the 5.9 mg Hg/ml pore water exposure, whilst the remaining had three replicates.

The substrate was prepared by mixing 4.5 g Sephadex G-100 with 40 ml artificial pore water and placed in 0.1 l plastic boxes. This was considered to be the approximately correct ratio of pore water and Sephadex, as it provided plenty of moisture and also allowed the worms to build tunnels that did not collapse. The exposure lasted 24 hours and the earthworms were observed for 3 days in clean soil. Survival was the only endpoint assayed.

2.3.8 Soil Pore water mercury concentration

This experiment was carried out to investigate the concentration of mercury in soil pore water. To collect the data needed, the chronic high-level exposure experiment was partially repeated. The original test setup was scaled down to using 50 g soil. 6 replicates for each concentration of mercury were used, each containing 1 earthworm with an average weight of 0.25 g.

2.4 Statistical analysis

Levenes' test was used to assess homogeneity of variance. Normally distributed results with sufficient replicates were analysed with one-way ANOVA and Dunnett's test. The non-parametric Mann-Whitney U test was used for the remaining results. The statistical analyses were performed on Statsoft Statistica 6.0. Graphs and regression analysis were created in GraphPad Prism 4. LC₅₀ values were calculated with ICPIN 2.0.

The regression curve fitted to mercury accumulation in earthworms from the exposures 2.3.5 and 2.3.6 was a one site binding hyperbola which has the equation: $Y=B_{\max} * X / (Kd + X)$.

This describes the binding of mercury to receptor sites that follow the law of mass action. B_{max} is the maximal binding, and Kd is the concentration of ligand required to reach half-maximal binding (description taken from GraphPad). Several regression approaches were tried, but this fitted best with the results.

Standard error of mean (SEM) and standard deviation (STD) are both used to describe variations in the results chapter, depending on the data presented and what kind of variation that needed to be communicated. The standard deviation was used to describe the variations seen in reproduction, growth, survival and burrowing speed. Standard errors were used to describe the uncertainties surrounding the mean values from the glutathione, protein and mercury assays (Altman and Bland 2005).

3 Results

3.1 Chronic and sub-chronic low-level exposures

Mercury concentrations ≤ 7.4 mg Hg/kg soil did not affect the growth of earthworms after two or four weeks of exposure (appendix Table 2). Cocoon production was significantly stimulated at 0.22 mg Hg/kg soil after two weeks (Fig 3.1.1), but was unaffected at other exposure concentrations after two and four weeks (appendix Table 1).

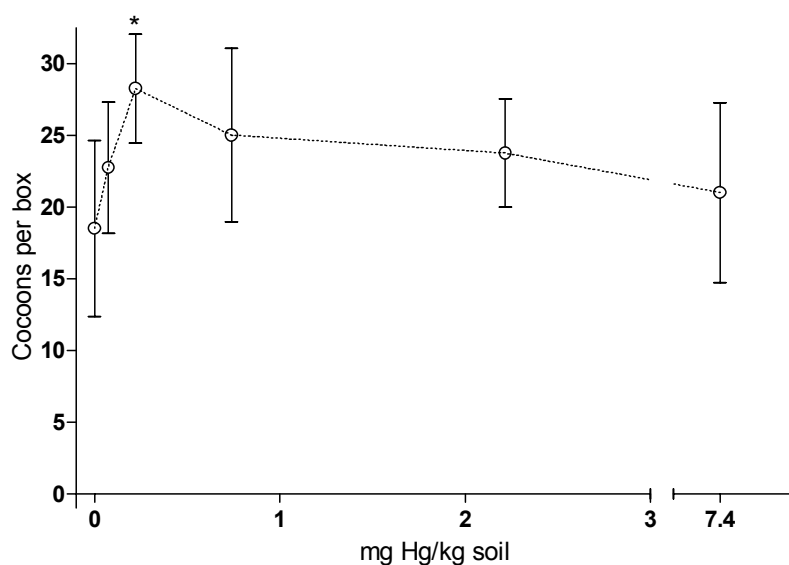


Fig 3.1.1 – Effect of Hg on earthworm (*E. fetida*) cocoon production after an exposure period of two weeks. Averages \pm STD are plotted. *indicates significant difference relative to controls (Mann-Whitney U test, $p < 0.05$, $n =$ four replicates with ten worms in each).

3.2 Sub-chronic intermediate-level exposure

Exposure concentrations up to 74 mg Hg/kg soil did not affect earthworm growth, GSH-level, burrowing speed or protein content (appendix Table 3,4 and 5). Mercury accumulation was significantly higher than controls in all exposures, and increased in a dose dependent manner (Fig 3.2.1).

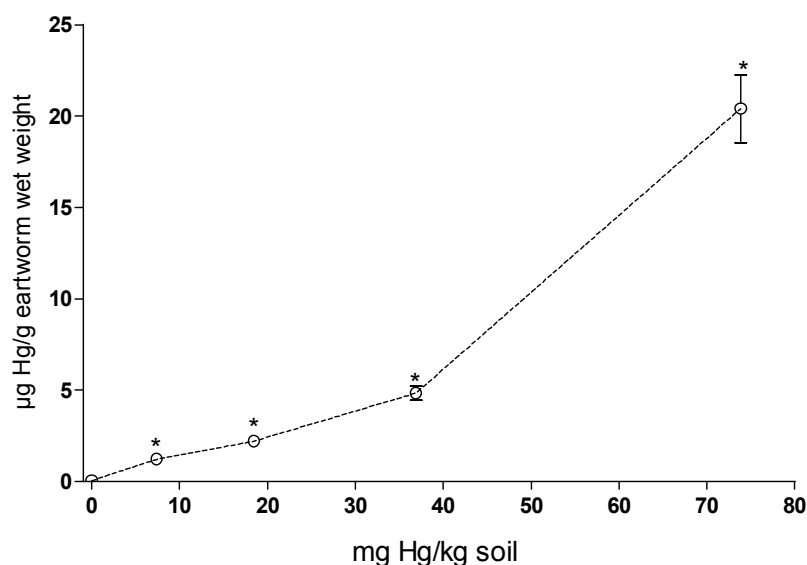


Fig 3.2.1 – Mercury accumulation in earthworms (*E. fetida*) after an exposure period of ten days in soil to various concentrations of mercury. Averages \pm SEM are plotted (for the lowest concentrations SEM is hidden by the points). *indicates significant difference relative to control (Mann-Whitney U test, $p < 0.05$, $n =$ three to six replicates with three worms in each).

3.3 Acute exposure and recovery trial

The burrowing speed and growth of earthworms was decreased when measured directly after the acute exposure and in earthworms that had recovered for 12 and 48 hours in clean soil. This was normalised again after 96 hours (Fig 3.3.1 and 3.3.2). Mercury levels in earthworms were significantly increased after the acute exposure and significantly reduced again after 96 hours (Fig 3.3.3). Earthworms exposed for 24 hours to 148 mg Hg/kg soil showed no mortality and no significant changes in GSH levels (Fig 3.3.4) or protein content (ANOVA $p > 0.05$) (appendix Table 6).

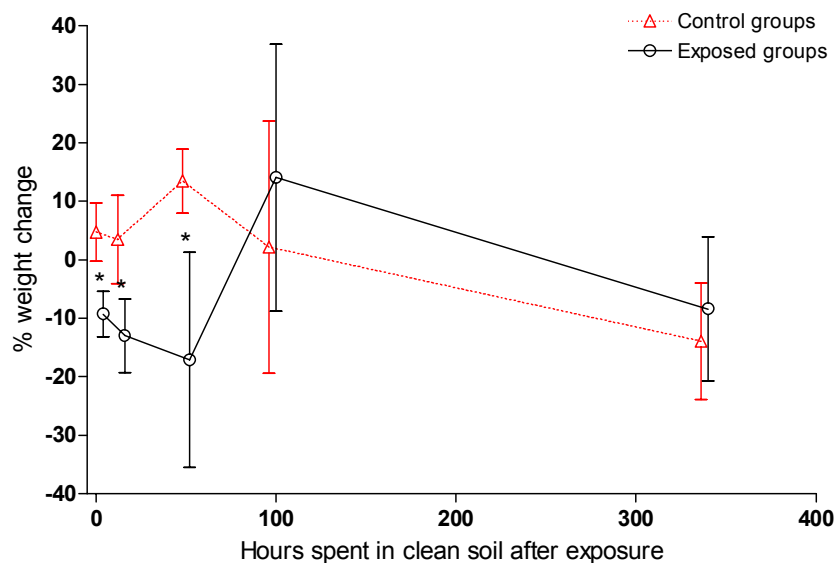


Fig 3.3.1 –Growth in earthworms (*E. fetida*) exposed for 24 hours. Figure shows average \pm STD. *indicates significant difference relative to control groups (Mann-Whitney U test, $p < 0.05$, $n =$ four replicates with five worms in each).

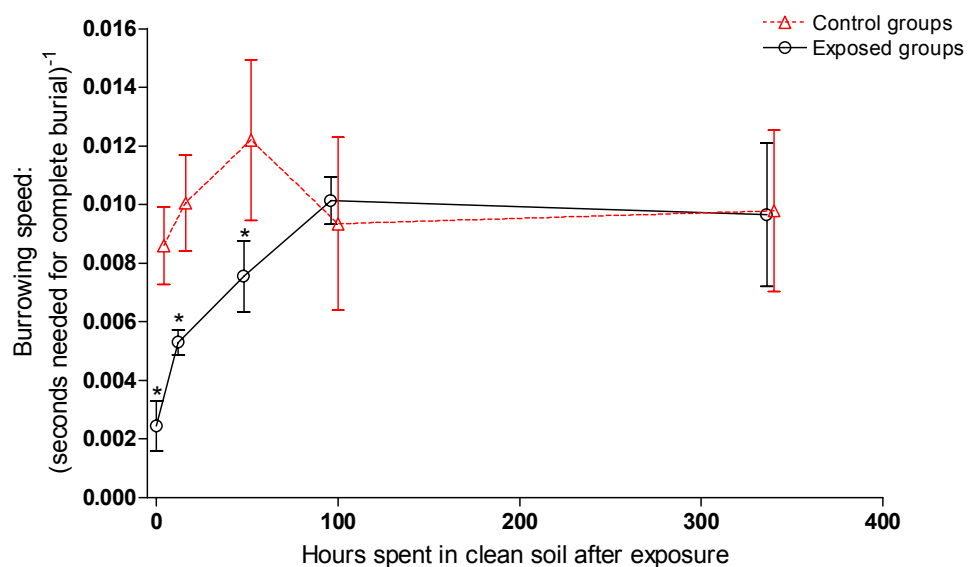


Fig 3.3.2 – Inverse of disappearance time \pm SEM (s^{-1}) for earthworms (*E. fetida*) exposed for 24 hours to 148 mg Hg/kg soil. Figure shows average \pm STD. *indicates significant difference relative to control groups (Mann-Whitney U test, $p < 0.05$, $n =$ four replicates with five worms in each).

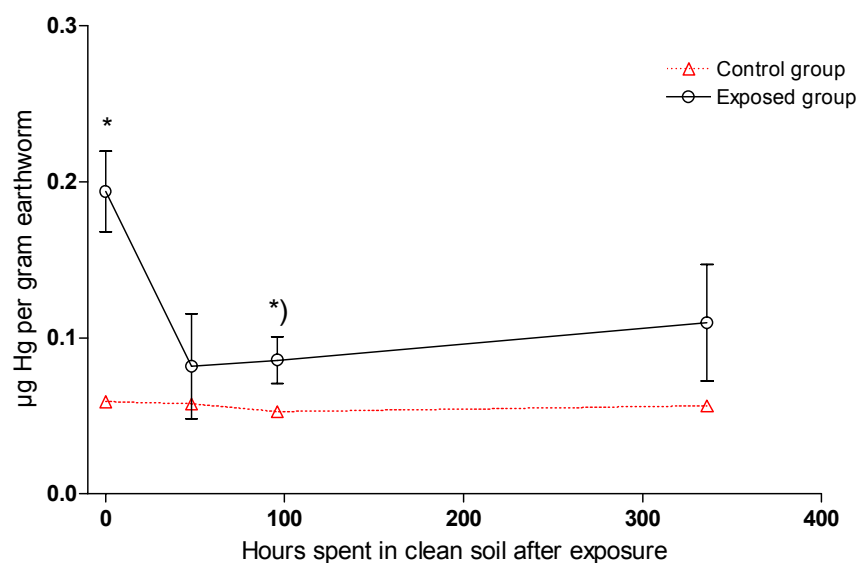


Fig 3.3.3 – Mercury accumulation and clearance in earthworms (*E. fetida*) exposed for 24 hours to 148 mg Hg/kg soil compared to control mercury levels. Figure shows average \pm SEM. * indicates significant difference relative to control, *) indicates significant difference relative to mercury values at time 0 (Mann-Whitney U test, $p < 0.05$, $n =$ six worms per time in clean soil).

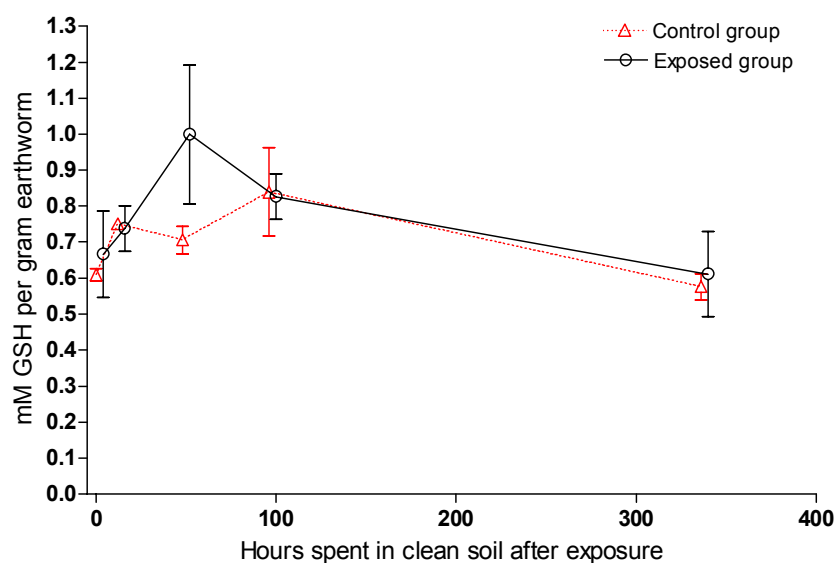


Fig 3.3.4 – GSH concentration in earthworms (*E. fetida*) exposed to 148 mg Hg/kg for 24 hours compared to control GSH levels. Figure shows average \pm SEM. No significant difference was detected when exposed earthworms were compared to the controls (Mann-Whitney U test, $p < 0.05$, $n =$ four replicates with five worms in each).

3.4 Chronic high concentration

Earthworm survival was significantly reduced at mercury concentrations ≥ 220 mg Hg/kg after a four week exposure period and had a LC_{50} of 355 mg Hg/kg (Fig 3.4.1). For the surviving earthworms, growth was significantly reduced compared to controls at concentrations ≥ 74 mg Hg/kg soil (Fig 3.4.2), cocoon production was completely arrested at mercury concentrations ≥ 22 mg Hg/kg (Fig 3.4.3), and burrowing speed was significantly decreased at concentrations ≥ 74 mg Hg/kg soil (Fig 3.4.4).

Tissue mercury concentrations were significantly increased relative to the controls at all exposure concentrations after the four week exposure period (Fig 3.4.5). The ratio of mercury concentration in earthworms relative to exposure concentration (i.e., biota-soil accumulation factor, BSAF) was close to 2 for concentrations up to 22 mg Hg/kg soil when linear regression was used. Higher concentrations were omitted from linear regression due to the earthworms' tendency to move out of the soil. The slope was 2.0, with a standard deviation of 0.13 and 95% confidence limits at 1.8 and 2.3.

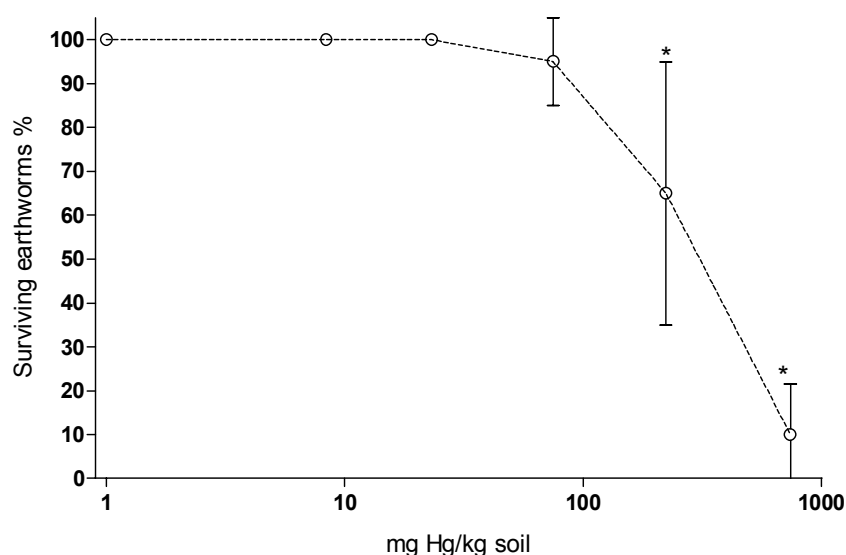


Fig 3.4.1 – Survival of earthworms (*E. fetida*) exposed to mercury for a period of four weeks. Figure shows average \pm STD. *indicates significant difference relative to control groups (Mann-Whitney U test, $p < 0.05$, $n =$ four replicates with five worms in each). Control concentrations were set to 1.0 to fit the Log scale.

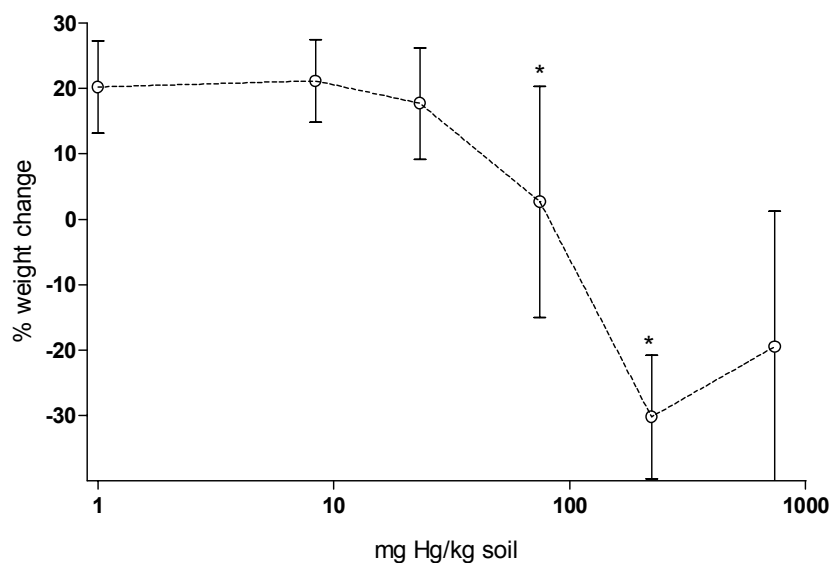


Fig 3.4.2 – Effect of Hg on earthworm (*E. fetida*) growth after an exposure period of four weeks. Figure shows average \pm STD. *indicates significant difference relative to controls (Mann-Whitney U test, $p < 0.05$, $n = 4$ (5 specimens each), lower n and number of specimens at the highest exposure levels due to reduced survival). Control concentrations were set to 1.0 to fit the Log scale.

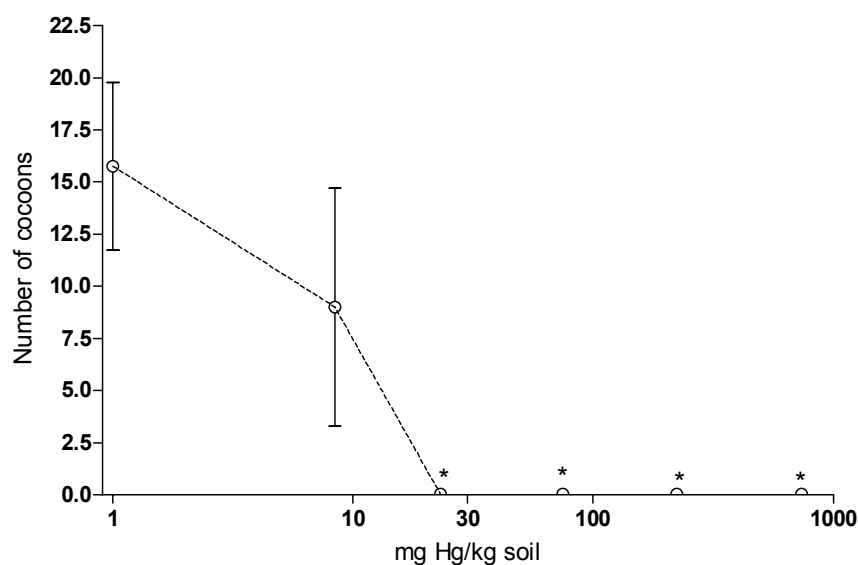


Fig 3.4.3 – Effect of Hg on earthworm (*E. fetida*) cocoon production after an exposure period of four weeks. Figure shows average \pm STD. *indicates significant difference relative to controls (Mann-Whitney U test, $p < 0.05$, $n = 4$ (5 specimens each), lower n and number of specimens at the highest exposure levels due to reduced survival). Control concentrations were set to 1.0 to fit the Log scale.

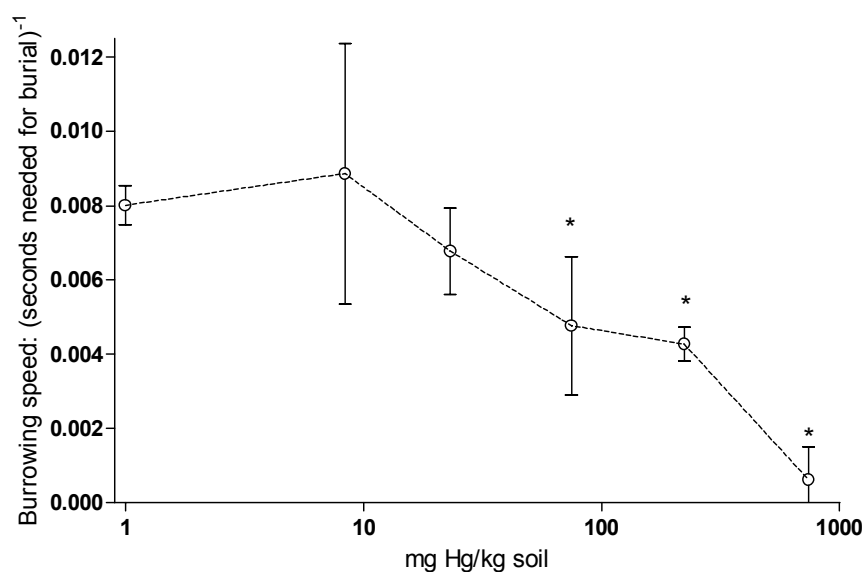


Fig 3.4.4 – Burrowing speed of earthworms (*E. fetida*) after exposure to mercury for a period of four weeks. Figure shows average \pm STD. *indicates significant difference relative to control groups (ANOVA and Dunnett's test, $p < 0.05$, $n = 4$ (5 specimens each), lower n and number of specimens at the highest exposure levels due to reduced survival). Control concentrations were set to 1.0 to fit the Log scale.

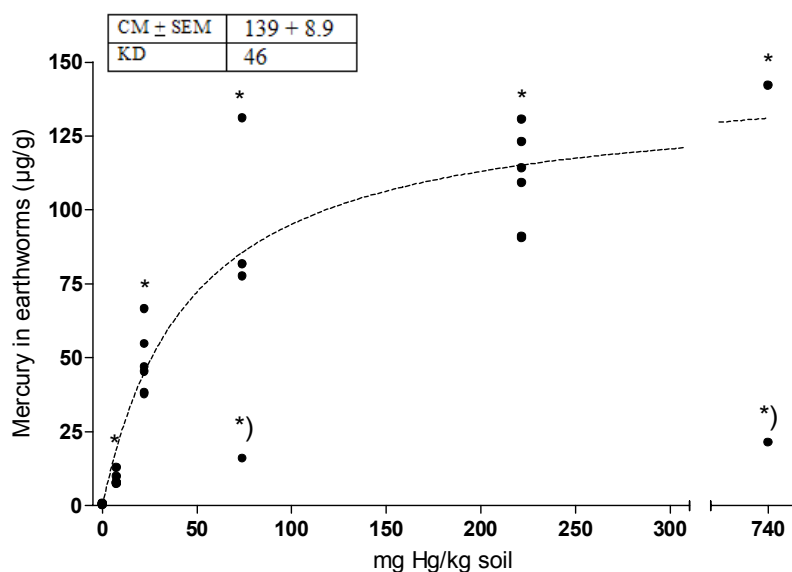


Fig 3.4.5 – Accumulation of mercury in earthworms (*E. fetida*) after an exposure period of four weeks. The points show individual worms. *indicates significant difference relative to control groups and *) indicates points omitted from statistical analysis as they represent earthworms not in contact with soil (Mann-Whitney U test, $p < 0.05$, $n =$ six earthworms per exposure concentration).

3.5 Effect of pre-exposure to mercury

The earthworms that were pre-treated with 22 mg Hg/kg soil for one week and thereafter kept one week in clean soil showed clear differences in survival (Fig 3.5.1), growth (Fig 3.5.2), reproduction (Fig 3.5.3), and mercury tissue concentrations (Fig 3.5.5) relative to the non pre-treated worms when exposed to high concentrations of mercury for four weeks. Glutathione levels were elevated in both the pre-exposed and non pre-exposed earthworms, but no difference between them was detected. LC₅₀ for pre-exposed and non pre-exposed worms were 545 and 170 mg Hg/kg soil, respectively.

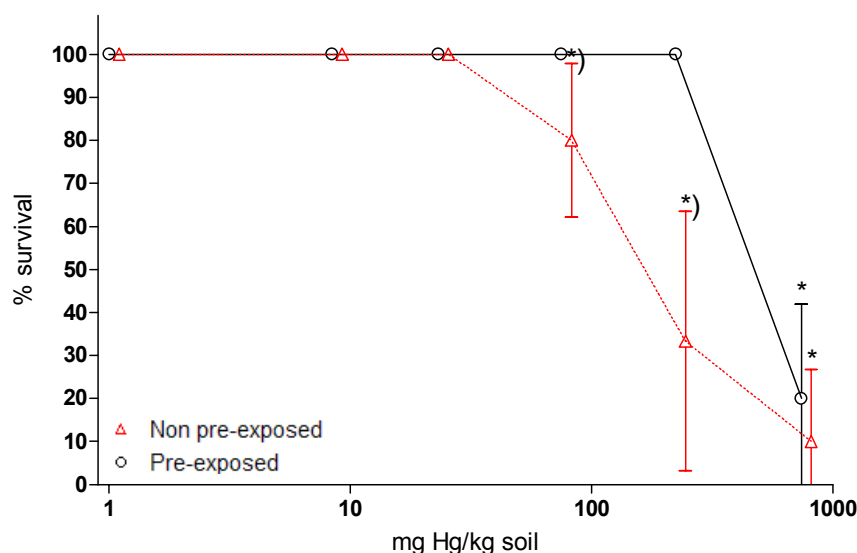


Fig 3.5.1 – Survival of earthworms (*E. fetida*) after an initial exposure to mercury (22 mg/kg – “pre-exposed”) or control soil (“non pre-exposed”) for a period of one week, and a second exposure to a range of mercury concentrations for a period of four weeks. Figure shows average \pm STD. * indicates significant difference relative to control groups, *) indicates significant difference between pre-exposed and non pre-exposed earthworms (Mann-Whitney U test, $p < 0.05$, $n =$ six replicates with five worms in each). Control concentrations were set to 1.0 to fit the Log scale.

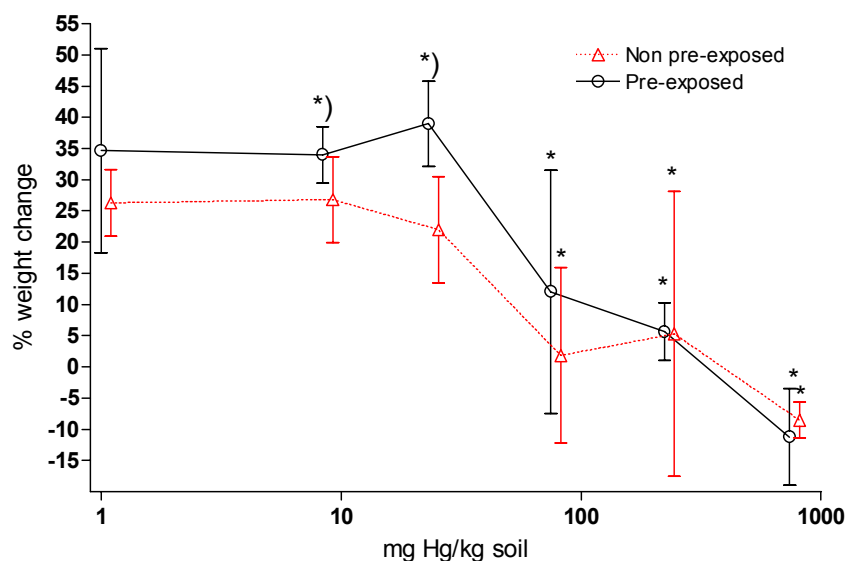


Fig 3.5.2 – The effect of mercury on earthworm (*E. fetida*) growth after an initial exposure to mercury (22 mg/kg – “pre-exposed”) or control soil (“non pre-exposed”) for a period of one week, and a second exposure to a range of mercury concentrations for a period of four weeks. Figure shows average \pm STD. * indicates significant difference relative to control, *) indicates significant difference between pre-exposed and non pre-exposed earthworms (ANOVA and Dunnett’s test, $p < 0.05$, $n = 6$ (5 specimens each), lower n and number of specimens at the highest exposure levels due to reduced survival). Control concentrations were set to 1.0 to fit the Log scale.

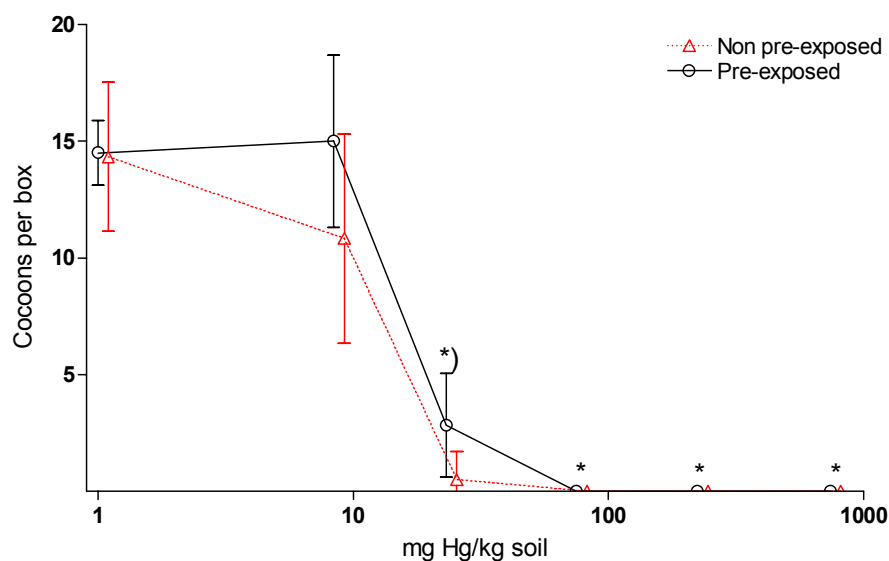


Fig 3.5.3 – Effect of Hg on earthworm (*E. fetida*) cocoon production after an initial exposure to mercury (22 mg/kg – “pre-exposed”) or control soil (“non pre-exposed”) for a period of one week, and a second exposure to a range of mercury concentrations for a period of four weeks. Figure shows average \pm STD. * indicates significant difference relative to controls, *) indicates significant difference relative to controls and significant difference between pre-exposed and non pre-exposed groups (Mann-Whitney U test, $p < 0.05$, $n = 6$ (5 specimens each), lower n and number of specimens at the highest exposure levels due to reduced survival). Control concentrations were set to 1.0 to fit the Log scale.

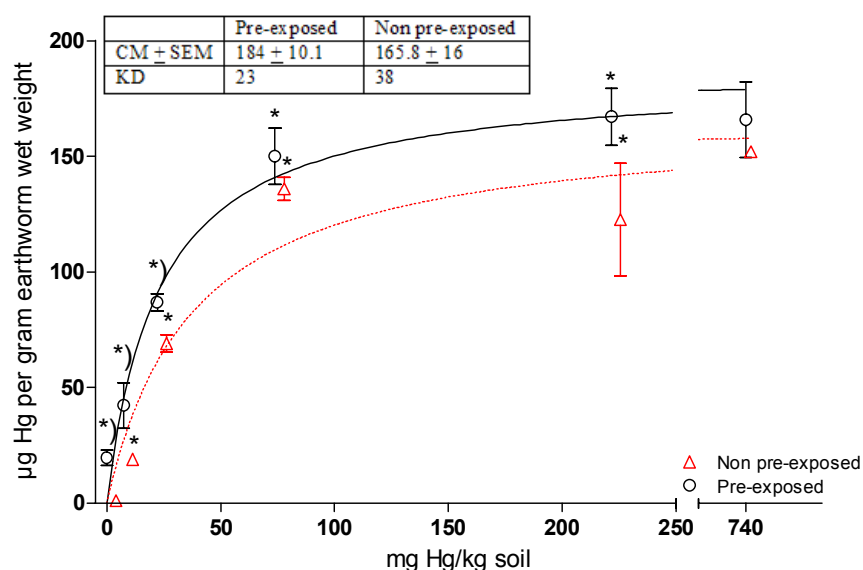


Fig 3.5.4 – Accumulation of mercury to earthworms (*E. fetida*) from soil after an initial exposure to mercury (22 mg/kg – “pre-exposed”) or control soil (“non pre-exposed”) for a period of one week, and a second exposure to a range of mercury concentrations for a period of four weeks. Figure shows average \pm SEM. * indicates significant difference relative to control groups, *) indicates significant difference relative to control groups and significant difference between pre-exposed and non pre-exposed earthworms. (Mann-Whitney U test, $p < 0.05$, $n = 6$ (5 specimens each), lower n and number of specimens at the highest exposure levels due to reduced survival).

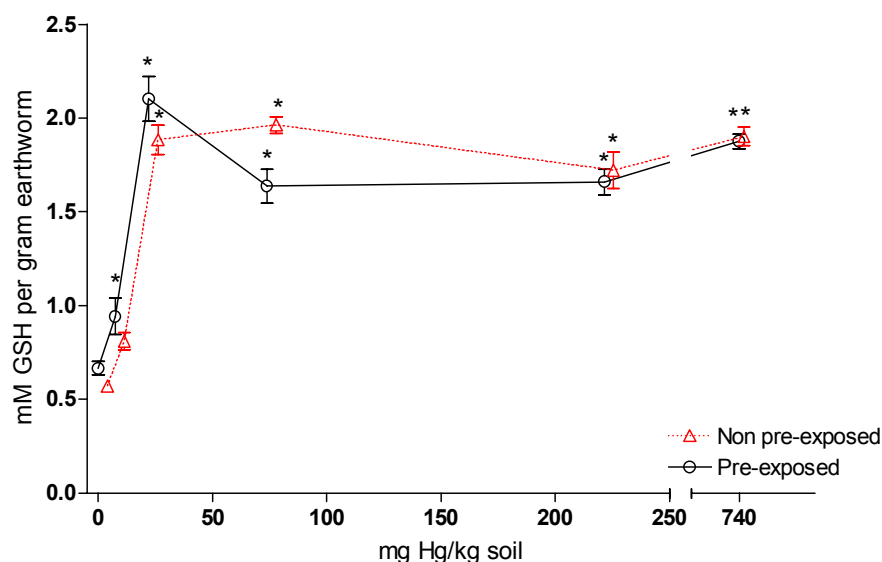


Fig 3.5.5 – The effect of mercury on earthworm (*E. fetida*) GSH levels after an initial exposure to mercury (22 mg/kg – “pre-exposed”) or control soil (“non pre-exposed”) for a period of one week, and a second exposure to a range of mercury concentrations for a period of four weeks. Figure shows average \pm SEM. * indicates significant difference relative to control (ANOVA and Dunnett’s test, $p < 0.05$, $n = 6$ (5 specimens each), lower n and number of specimens at the highest exposure levels due to reduced survival).

3.6 Earthworm toxicity testing in an artificial substrate (Sephadex)

Survival was significantly affected in earthworms exposed to mercury for 24 hours in Sephadex G-100 gel. As the concentration of mercury increased from 4.4 to 6.7 mg Hg/L, mortality increased and then decreased again. Earthworms were observed outside the exposure substrate at 7.0 and 7.4 mg Hg/L. Significant mortality relative to the controls was observed in the concentrations 6.7 and 7.0 mg Hg/L (Fig 3.6.1).

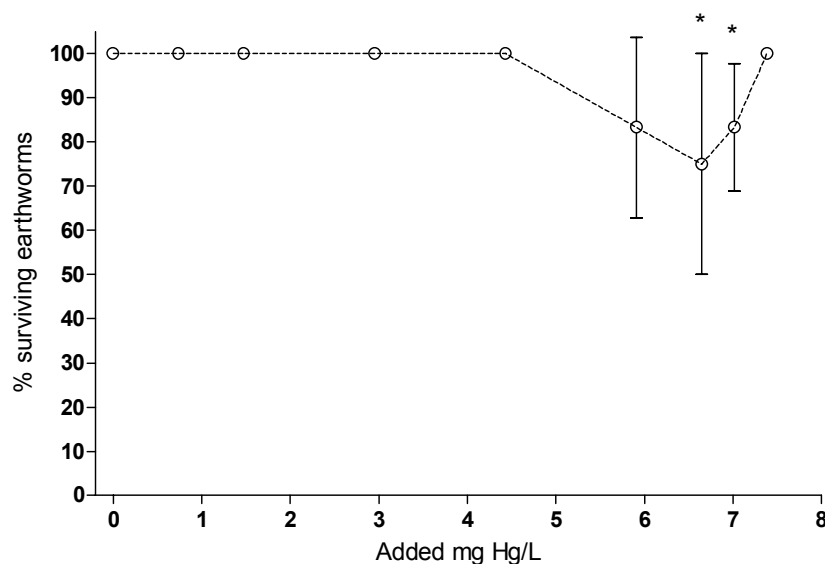


Fig 3.6.1 – Earthworm (*E. fetida*) survival three days after a 24 hour exposure period in Sephadex G-100 gel. Figure shows average \pm STD. *indicates significant difference relative to control groups (Mann-Whitney U test, $p < 0.05$, $n = 3$ to 6).

3.7 Soil pore water mercury concentration

Due to instrument failure, results from this experiment were not reliable. Thus, results from this experiment will not be discussed.

4 Discussion

The objective of this thesis was to investigate the effects of mercury exposure on the earthworm *E. fetida*. The main findings were that pre-exposure reduced the adverse effects of mercury and that glutathione levels were elevated in response to increased tissue levels of mercury.

The non-destructive endpoints growth, reproduction, burrowing speed and survival were used in order to make the most of the experiments performed. The setup for chemical analysis was also tailored to enable glutathione, protein and mercury analysis to be performed on the same worms, thus increasing the amount of data relative to the total number of earthworms. Together these tests gave a good indication of how earthworms respond to mercury exposure both in relation to accumulation patterns, biochemical responses, burrowing behaviour, and with respect to energy allocation (growth and reproduction).

4.1 Effect levels for various endpoints

4.1.1 Survival

Survival was the least sensitive parameter and was only affected at the highest exposure concentrations after four weeks exposure (Figs. 3.4.1 and 3.5.1). Fischer and Koszorus (1992) found an LC_{50} -value for earthworms at 100 mg $HgCl_2$ /kg wet weight peaty marshland. The scarce information provided on soil properties made it difficult to compare the observation to the LC_{50} -values 170 and 355 mg Hg/kg soil found in two experiments in this thesis. One artefact on survival measurements was that some earthworms survived high-level exposures by regularly crawling out of the exposure medium. This behaviour decreased their contact with the contaminated soil. A similar effect was observed in the Sephadex exposure, where mortality in the highest concentration was decreased compared to lower ones, due to exposure avoidance (Fig 3.6.1). This behaviour has been seen for earthworms with many substances (Gilman and Vardanis 1974; Yeardeley et al. 1996; Slimak 1997; Langdon et al. 2001; Vermeulen et al. 2001; Schaefer 2004).

4.1.2 Growth

Effects on earthworm growth were observed at relatively high concentrations of mercury (≥ 74 mg Hg/kg soil). The growth measurements of earthworms had a relatively large variation in the acute exposure experiment (Fig 3.3.1), probably because the earthworms were not depurated prior to weighing. The contribution from the gut content to the total weight in the individual earthworms may have been substantial, but fairly constant during the experiment. Measurements related to the weight of earthworms (i.e., protein content, GSH-concentration, growth, and mercury absorption) from this experiment should therefore be considered relative to the controls. A few individuals lost their tails, and this also contributed to the large variation in growth.

Earthworm growth was adversely affected after four weeks of exposure to mercury concentrations ≥ 74 mg/kg (Figs. 3.4.2 and 3.5.2). Conclusions are difficult to draw from the highest exposure concentration because of the high mortality. The surviving worms may have had weights that deviated considerably from the group average. Kokta (1992) concludes that results on earthworm growth should only be used when less than 20% mortality occurs. However, statistical analysis was performed, as they may be of interest for further work.

4.1.3 Reproduction

While low levels of mercury (0 – 7.4 mg Hg/kg soil) in the sub-chronic experiment did not affect production of juveniles after two or four weeks, cocoon production was significantly higher ($p < 0.05$) at 0.22 mg Hg/kg soil than in control groups after two weeks (Fig 3.1.1). The observed elevation of cocoon production at low, sub-lethal concentrations of mercury may be an indication of hormesis, which is defined as a modest overcompensation to a disruption in homeostasis (Calabrese and Baldwin 2001). Although the increased cocoon production was statistically significant, the four-week data at the same exposure level showed no such effect, indicating that the experiment might benefit from being repeated before a safe conclusion about the presence or absence of hormesis can be made. Studies conducted by Fischer and Koszorus (1992) gave similar results, but at higher concentrations. They demonstrated

significantly enhanced cocoon production by earthworms at 100 mg Hg/kg peaty marshland soil after two weeks, which is also the LC₅₀ in their experiments.

Reproduction turned out to be the most sensitive endpoint in the chronic exposures (Figs. 3.4.3 and 3.5.3). This is in line with the findings of Lock and Janssen (2001) who reported a considerable reduction of cocoon production at 18 mg Hg/kg OECD soil relative to controls. The OECD soil and the Ås soil have similar pH-values (pH 6.0 and 6.2, respectively) and cation exchange capacities (141 mmol(+) kg⁻¹ and 120 mmol(+) kg⁻¹, respectively). Van Gestel (1992) reviews that these are the two most important parameters for the bioavailability of the heavy metals cadmium, lead and zinc in soil. As mercury has many of the same chemical properties as these metals, this suggests that the two parameters can also be critical for the availability of mercury. Thus, similar results from mercury exposure can be expected in the two soils.

4.1.4 Burrowing speed.

The endpoint burrowing speed had a high level of uncertainty, which is probably an inherent property of this parameter. This can to some degree be attributed to the fact that some worms tended to halt their burrowing for some time, with the tip of the tail still visible. After the acute exposure, the earthworms showed reduced speed, but this effect ceased 96 hours after transfer to clean soil (Fig 3.3.2). The ability of earthworms to burrow into soil was reduced after four weeks exposure to concentrations ≥ 74 mg Hg/kg (Fig 3.4.4). This could have been caused by a direct effect of mercury on the earthworms. But, as the earthworms were mostly sedentary during exposure at these concentrations, they were likely to eat less than worms in lower concentrations. Lack of nutrition might have influenced the results from the chronic exposure, but was not likely to have an effect on the burial performance in the acute exposure.

Burrowing speed has previously been used by Stenersen (1981), who showed that several acetylcholineesterase-inhibiting pesticides increased the disappearance time. Adverse affects on growth and burial time correlated well in the chronic high-level

exposures and the acute exposure. As they both serve as indicators of earthworm health, this strengthens the validity of burrowing speed as an endpoint.

4.1.5 Glutathione levels

GSH concentrations were greatly increased in earthworms exposed to intermediate and high concentrations of mercury for four weeks (Fig 3.5.5). Lawson (2003), reported that glutathione levels were increased in earthworms in response to SOD inhibition by fluoride. Increased levels of glutathione has been observed in mouse macrophage like cell cultures after exposure to SNP, which contributes to oxidative stress through acting as a NO donor and its iron moiety (Kurozumi and Kojima 2002; Kurozumi et al. 2003). The initial hypothesis was that GSH could be a protective substrate against mercury that would be depleted as a result of exposure. This is supported by the results presented by Nielsen et al. (1991), who found decreased GSH levels when exposed to high doses of mercury in rat kidneys. It also ties in well with the findings of Lund et al. (1993), who reported decreased levels of GSH in rat kidney mitochondria.

In light of the findings from the experiments performed in this thesis, it was clear that GSH levels were elevated after exposure to mercury. Mercury accumulation and glutathione concentrations in earthworms exposed to high concentrations followed the same pattern (Fig 3.5.4 and Fig 3.5.5). This suggests that glutathione concentrations and mercury accumulation are closely related, and GSH levels may therefore be an interesting biomarker for mercury after chronic exposure.

Stokke and Stenersen (1993) demonstrated that earthworm glutathione transferases were non-inducible and constituted approximately 0.6% of total cytosolic protein in the earthworm *Eisenia andrei*. The results presented in this thesis showed that production of glutathione was increased in earthworms exposed to mercury. As there is a considerable amount of glutathione transferase constitutively present in earthworms, it is a plausible hypothesis that glutathione levels, but not glutathione transferases, are regulated in response to oxidative stress.

The assays for acid-precipitated protein content in the earthworms never yielded significant results when comparing the exposed groups to the controls. However, changes were not expected. The protein assays were performed in an attempt to correlate them with glutathione levels. No such correlation was found between GSH concentration and protein in earthworms from the acute exposure (appendix Table 6).

4.2 Mercury accumulation patterns

After a ten day exposure to intermediate levels, an exposure-related accumulation of mercury was observed. However, accumulation was much greater at the highest concentration compared to the other exposure concentrations (Fig 3.2.1). This may be explained by saturation of the soil with mercury, which leaves more mercury available for accumulation to earthworms.

A second explanation may be loss of the ability to regulate metal accumulation. Van Gestel and van Diepen (1997) observed that accumulation in the collembola *Folsomia candida* exposed to low concentrations of cadmium reached a plateau after two weeks, but continued to rise when exposed to higher levels. They encourage caution when interpreting these results, due to the considerable mortality observed at high cadmium levels. However, their observations support the hypothesis that the increased accumulation observed at 74 mg Hg/kg could be a result of reduced ability to regulate the internal metal accumulation. The internal mercury concentrations were lower than in the earthworms that exhibited effects on burrowing speed, glutathione concentration and growth after four weeks in similar exposure concentrations (Fig 3.2.1, 3.4.5 and 3.5.4), which explains the lack of adverse effects in the sub-chronic intermediate-level exposure.

The earthworms exposed to a single high concentrations of mercury for 24 hours showed an increased amount of mercury relative to controls, which was decreased again shortly after transfer to clean soil (Fig 3.3.3). As the earthworms in this experiment were not depurated, it is not known whether the rapid decrease could be attributed to their gut content or if mercury was excreted from internal compartments.

The mercury accumulation in earthworms exposed to high concentrations for four weeks, reached a plateau at 139 ± 9 and 165 ± 16 $\mu\text{g Hg/g}$ earthworm wet weight in two separate experiments (Figs. 3.4.5 and Fig 3.5.4). A similar plateau-shaped cadmium accumulation is observed in collembola by Van Gestel and Hensbergen (1997). Above this plateau all animals died, an observation that ties in well with the high mortality observed in the highest exposure concentrations used in this thesis. Accumulation of mercury in earthworms was probably the assay in which behaviour had the largest impact. Exposure avoidance at high concentrations caused reduced accumulation and subsequently large variations in tissue mercury levels (Fig 3.4.5), and some earthworms were omitted from the regression curves as a result of this.

4.3 Critical body burdens for observation of effects

Measurements of internal mercury concentrations in combination with other measured endpoints can be used to assess the critical body burden for the various responses. The critical body burdens were based on the two chronic high concentration experiments.

The rapid post mortem degradation of tissue in soil made mercury analysis in earthworms impossible. Therefore, the data on lethal mercury tissue levels were not obtained, but it seems plausible that earthworms do not survive tissue concentrations above 139 and 165 $\mu\text{g Hg/g}$ earthworm wet weight, which were the highest average concentrations measured in the two experiments.

Significant effects on growth were seen in earthworms with 106 and 136 $\mu\text{g Hg/g}$ earthworm wet weight in the two experiments, while reproduction was arrested in earthworms that contained 48 and 71 $\mu\text{g Hg/g}$ earthworm wet weight. Burrowing speed was decreased in worms containing 106 $\mu\text{g Hg/g}$ earthworm wet weight.

4.4 Effects of pre-exposure to mercury

The pre-treated earthworms were much more tolerant to mercury exposure. This was evident from the survival (Fig 3.5.1), growth (Fig 3.5.2) and reproduction (Fig 3.5.3) studies conducted. The pre-exposed worms were less affected than the non pre-exposed earthworms at the same exposure concentrations, although they had higher internal concentrations of mercury (Fig 3.5.4). These results indicate that hormesis

has occurred as a response to the pre-exposure (E.J. Calabrese – personal communication 14. October 2005). Reduced mortality has also been shown for the enchytraeid *Enchytraeus buchholzi* after pre-treatment with cadmium and subsequent exposure to copper (Willuhn et al. 1996).

The observations and results in the chronic high-level exposure experiment were to a large extent reproduced for the non pre-treated earthworms in this experiment, and shows reproducibility of the setup. As no differences in glutathione concentrations were found between pre-exposed and non pre-exposed earthworms, glutathione cannot explain the differences seen with respect to survival, burrowing speed, growth and reproduction.

Mercury concentrations were significantly higher in the pre-exposed than the non pre-exposed earthworms at low exposure levels (0 – 22.2 mg Hg/kg soil), but not at the higher levels (Fig 3.5.4). The elevated levels in pre-exposed earthworms can be explained as remnants from the pre-exposure, which were camouflaged by variation at higher levels. The plateau-shaped absorption observed in the pre-treated and the non pre-treated earthworms, having maximum of $184 \pm 10 \mu\text{g/g}$ and $165 \pm 16 \mu\text{g Hg/g}$ earthworm wet weight respectively, showed that pre-exposed earthworms do not excrete more mercury than the non pre-exposed. However, mercury that is sequestered through association with metallothioneins in earthworm tissue will still influence the readout of mercury concentrations.

Homa et al. (2005) report an increase in earthworm MT2, HSP70 and HSP72 after a three day exposure to the heavy metals cadmium, lead, copper and zinc. Mercury is known to bind and also increase the production of metallothionein in other organisms, suggesting that this may also be the case for earthworms. (Vasak et al. 1981; Roesijadi 1992; Palmiter 1994; Wautier 2000; Geret 2002; Beattie 2005; Wang and Rainbow 2005). An elevated level of metallothionein in earthworms after exposure to mercury could explain the observed difference between pre-treated and non pre-treated earthworms.

4.5 Observed, not quantified effects of exposure

After 24 hours, exposed earthworms were more sensitive than usual and reacted to gentle handling in the same way they normally would to being treaded on a fishing-hook.

Earthworms exposed to high concentrations (e.g. 74 – 740 mg Hg/kg soil) did not curl up when frozen. While control groups were mostly nestled in a small ball in the bottom on their cryo-tube, earthworms from higher concentrations were often stretched out the length of the tube. Coiling is observed in the earthworms *Eisenia nordenskioeldi* entering estivation (hibernation) when temperatures drop, suggesting that mercury reduced the adaptability of earthworms to environmental changes (Holmstrup et al. 1999).

When frozen, earthworms exposed high levels of mercury excreted coelomic fluids, a behaviour observed by Holmstrup et al. in the freeze-intolerant earthworm species *Lumbricus rubellus* and *Aporrectodea caliginosa* when frozen (Holmstrup et al. 1999).

Earthworms that seemed to be nearly dead, sometimes recovered. Some that had lost everything from the clitellum and down even regrew tails to some extent. Almost all such damage seemed to begin near the anus. Stenersen (1979) observed that in earthworms exposed to pesticides, the front end of the earthworm still reacted to stimuli, while most of the body was rotten.

Earthworms exposed to high concentration of mercury generally ate less than those in lower levels, even though they were often found in the horse manure provided as food. The manure did not disappear in the highest levels of mercury, while most or all was gone after two weeks in the lowest concentrations.

Some of the earthworms were blotched, having areas without pigment. This was observed in higher concentrations (e.g. 74 – 740 mg Hg/kg soil) and in most cases at

the front end of the earthworms. The earthworms did not seem to be in worse shape than worms exposed to the same concentrations that did not have this loss of pigment.

4.6 Conclusions

Pre-exposure to mercury (22 mg/kg soil for one week) strongly increased survival in a subsequent standard test carried out one week after the pre-treatment, whereas differences between pre-exposed and non pre-exposed worms for cocoon production and growth were smaller, though statistically significant.

Earthworms exposed to mercury (22 - 740 mg/kg soil for four weeks) experienced a threefold increase in glutathione levels from a background level of approximately 0.62 $\mu\text{mol/g}$. Glutathione levels were similar in pre-exposed and non pre-exposed earthworms, suggesting that increased glutathione levels did not cause the increased survival in pre-exposed animals. The shape of the curves for mercury accumulation resembled the shape of the curves for glutathione concentrations in earthworms when plotted against mercury concentrations in soil, suggesting that glutathione levels and internal mercury concentrations in earthworms are closely related.

4.6.1 Suggestions for further work

Results presented here fail to explain the reason for the increased resistance that pre-exposed earthworms showed towards mercury. Metallothionein levels were not assayed and may be an interesting protein for assays in further research. An elevated level of metallothionein in earthworms after exposure to mercury could explain the observed difference between pre-treated and non pre-treated earthworms.

Due to time constraints, mercury and glutathione content of different tissues in earthworms was not analyzed. This might shed some light on whether mercury was deposited differently in pre-exposed earthworms and whether the metal was co-allocated with glutathione.

An increased GSH concentration, as a response to agents causing oxidative stress, has been observed in many cases. To expand the knowledge of the connection between

oxidative stress and glutathione levels in earthworms, it is suggested that the classical ROS producing substance paraquat is assayed for its ability to induce production of glutathione.

Analysis of mRNA expression for enzymes involved in the synthesis and maintenance of GSH levels, such as glutathione reductase and γ -glutamylcysteine synthetase, or indeed the abundance of these enzymes in earthworm tissues after an exposure to mercury, would be valuable additions to the results from this thesis.

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Appendix

Table 1: Chronic and sub-chronic low-level exposures; reproduction data

mg Hg/kg soil	Cocoons at 4 weeks	Juveniles at 2 weeks	Juveniles at 4 weeks
0	75	87	156
0	49	56	161
0	73	101	132
0	74	45	153
0.074	63	83	147
0.074	53	78	186
0.074	51	111	101
0.074	60	82	140
0.22	71	93	160
0.22	58	107	123
0.22	67	108	133
0.22	66	65	117
0.74	67	102	161
0.74	73	67	159
0.74	76	101	178
0.74	66	63	140
2.2	50	95	129
2.2	53	90	145
2.2	50	96	126
2.2	59	84	113
7.4	53	67	107
7.4	51	55	134
7.4	73	49	169
7.4	55	43	124

Table 2: Chronic and sub-chronic low-level exposures; weight change

mg Hg/kg soil	Two weeks			Four weeks		
	Initial weight (g)	After exposure (g)	% change	Initial weight (g)	After exposure (g)	% change
0	0.39	0.39	0,26	0.26	0.35	33.08
0	0.38	0.36	-4,26	0.29	0.34	18.34
0	0.39	0.41	4,87	0.29	0.36	24.40
0	0.38	0.35	-8,12	0.29	0.36	24.40
0.074	0.39	0.39	1,04	0.30	0.36	20.95
0.074	0.4	0.4	0	0.31	0.38	23.53
0.074	0.39	0.35	-8,76	0.31	0.38	21.29
0.074	0.40	0.35	-11,34	0.30	0.38	28.14
0.22	0.36	0.34	-6,94	0.30	0.41	34.77
0.22	0.41	0.43	4,13	0.30	0.36	22.37
0.22	0.38	0.40	4,47	0.28	0.32	13.03
0.22	0.34	0.35	3,87	0.30	0.37	21.67
0.74	0.36	0.36	0,83	0.31	0.38	22.01
0.74	0.38	0.38	0,8	0.29	0.36	25
0.74	0.38	0.39	2,62	0.32	0.37	13.31
0.74	0.37	0.37	0,54	0.30	0.35	18.79
2.2	0.36	0.31	-15,04	0.29	0.38	31.49
2.2	0.40	0.38	-5,49	0.28	0.33	18.77
2.2	0.35	0.37	6,63	0.34	0.37	8.53
2.2	0.4	0.38	-4,5	0.32	0.41	27.36
7.4	0.37	0.39	5,14	0.28	0.37	32.49
7.4	0.36	0.41	12,40	0.28	0.35	22.26
7.4	0.36	0.35	-4,17	0.30	0.4	33.33
7.4	0.39	0.38	-0,78	0.24	0.34	43.93

Table 3: Sub-chronic intermediate-level exposure; GSH and protein levels

mg Hg/kg soil	μmol GSH/g earthworm	mg Prot/g earthworm
0	0.19	2.92
0	0.11	2.98
0	0.12	3.50
0	0.13	2.92
0	0.25	2.14
0	0.15	2.81
7.4	0.23	2.92
7.4	0.08	2.80
7.4	0.09	3.95
18.5	0.15	3.20
18.5	0.20	3.30
18.5	0.18	3.61
37	0.18	3.43
37	0.33	3.47
37	0.14	4.00
37	0.06	3.86
74	0.09	3.49
74	0.20	3.04
74	0.18	4.09
74	0.42	3.55

Table 4: Sub-chronic intermediate-level exposure; Burrowing time

mg Hg/kg soil	before exposure (s)	after exposure (s)	% change
0	122	131	8
0	150	192	28
0	102	145	43
0	107	132	23
0	113	120	6
0	100	111	11
7.4	120	141	18
7.4	119	89	-25
7.4	126	108	-14
18.5	143	119	-17
18.5	108	142	31
18.5	135	171	26
37	92	135	46
37	128	138	8
37	150	155	4
37	125	139	11
74	78	136	74
74	112	139	24
74	133	155	17
74	121	194	61

Table 5: Sub-chronic intermediate-level exposure; weight change

mg Hg/kg soil	Initial weight (g)	Weight after exposure (g)	% change
0	0.33	0.25	30.91
0	0.29	0.28	4.79
0	0.30	0.26	13.46
0	0.28	0.27	2.44
0	0.31	0.30	4.52
0	0.30	0.27	10.43
7.4	0.30	0.26	14.40
7.4	0.30	0.27	9.88
7.4	0.28	0.25	7.89
18.5	0.28	0.25	10.74
18.5	0.28	0.28	0
18.5	0.28	0.26	5.73
37	0.27	0.27	1.25
37	0.29	0.26	8.86
37	0.29	0.27	6.17
37	0.26	0.27	-1.26
74	0.27	0.26	5.13
74	0.30	0.29	3.51
74	0.25	0.24	6.34
74	0.28	0.26	9.03

Table 6: Acute exposure and recovery: GSH and protein levels

Hours after exposure	mg Protein/g earthworm		μmol GSH/g earthworm	
	exposed	controls	exposed	controls
0	4.54	3.38	0.64	0.88
0	5.13	4.40	0.59	0.71
0	4.67	4.08	0.64	0.90
0	1.46	3.42	0.57	0.72
12	1.81	4.65	0.75	0.33
12	1.42	3.95	0.77	0.71
12	4.11	2.64	0.76	0.68
12	3.01	4.23	0.72	1.21
48	4.95	4.41	0.71	0.80
48	1.09	4.25	0.81	0.51
48	3.11	3.92	0.67	0.76
48	3.86	4.42	0.64	0.92
96	2.67	3.07	0.47	0.50
96	3.16	3.74	1.00	0.77
96	3.49	3.25	0.94	0.74
96	3.59	4.05	0.94	0.32
336	3.60	2.89	0.57	0.64
336	2.62	5.04	0.57	1.39
336	3.77	5.03	0.67	1.01
336	4.37	4.52	0.50	0.86